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Phospho-mimicking Atf1 mutants bypass the transcription activating function of the MAP kinase Sty1 of fission yeast

Laura Sánchez-Mir[1†], Clàudia Salat-Canela[1,†], Esther Paulo[1,3], Mercè Carmona[1], José Ayté[1], Baldo Oliva[2] and Elena Hidalgo[1*]

[1]Oxidative Stress and Cell Cycle Group and [2]Structural Bioinformatics Laboratory (GRIB), Universitat Pompeu Fabra, C/ Dr. Aiguader 88, 08003 Barcelona, Spain

[†]These authors contributed equally to this work

[3]Present address: Cardiovascular Research Institute, Department of Physiology, University of California, San Francisco, 555 Mission Bay Blvd. South, San Francisco CA, 94158

*For correspondence: elena.hidalgo@upf.edu

ABSTRACT

Stress-dependent activation of signaling cascades is often mediated by phosphorylation events, but the exact nature and role of these phosphorelays are often poorly understood. We analyze here which are the consequences of the stress-dependent phosphorylation of a transcription factor on gene activation. In fission yeast, the MAP kinase Sty1 is activated upon several environmental hazards and promotes cell adaptation and survival, greatly through activation of a gene program mediated by the transcription factor Atf1. Although described decades ago, the role of the phosphorylation of Atf1 by Sty1 is still a matter of debate. We present here a brief review of recent data, obtained through the characterization of several phosphorylation mutant derivatives of Atf1, demonstrating that Atf1 phosphorylation does not stabilize the factor nor stimulates its binding to DNA. Rather, it provides a structural platform of interaction with the transcriptional machinery. Based on these findings, future work will establish how this phosphorylated trans-activation domain promotes the massive gene expression shift allowing cellular adaptation to stress.

Keywords:

transcription regulation / *Schizosaccharomyces pombe* / phosphorylation / Sty1 / Atf1 / oxidative stress

Introduction

Signal transduction, the process by which environmental or endogenous signals invoke a series of events meant to induce cellular adaptation or survival, are frequently based on phosphorelay systems. The downstream effectors of these cascades are often transcription factors, which upon phosphorylation by a signal-activated protein kinase suffer a gain-of-function which facilitate a change in the gene expression program. Once determined that a transcription factor is phosphorylated in a signal-dependent manner, decades of intensive work can be required to define the molecular events driven by this post-translational modification.

Eukaryotic organisms respond to environmental cues by triggering stress-dependent gene expression programs driven by MAP kinase pathways (Ho and Gasch 2015). Upon signal activation, the MAP kinase is phosphorylated and travels to the nucleus to trigger phosphorylation of transcription factors. Once the transcription factors are activated, promote specific cellular responses to extracellular signals by adapting the complex transcriptional machinery into particular sets of genes. Thus the gene expression program is modified by cofactors of RNA polymerase II, transcription factors, histone modifying enzymes, histone variants or chromatin remodelers (Weake and Workman 2010). In fission yeast, *Schizosaccharomyces pombe,* the Sty1 MAP kinase pathway responds to different types of environmental stresses to allow survival. The pathway is induced by most stress conditions, and triggers a wide transcriptional shift of up to 5-15% of the yeast genome (Chen, et al. 2003, Chen, et al. 2008). Upon stress, Sty1 is phosphorylated and transiently accumulates at the nucleus, where it promotes transcriptional regulation of genes in, at least partially, an Atf1-dependent manner (Millar, et al. 1995, Shiozaki and Russell 1995, Shiozaki and Russell 1996, Wilkinson, et al. 1996). Atf1 is a basic zipper (bZIP)-containing transcription factor which heterodimerizes with Pcr1, another bZIP protein (Lawrence, et al. 2007). Although they have overlapping functions (Sanso, et al. 2008), Atf1 seems to be the direct target of the MAP kinase Sty1 (Wilkinson, et al. 1996). To trigger both nuclear accumulation as well as activation of its kinase activity, Sty1 has to be phosphorylated, since neither Atf1 is phosphorylated, nor transcription is activated in a constitutive nuclear version of the kinase (Castillo, et al. 2003, Sanso, et al. 2008). In response to extracellular hydrogen peroxide (H_2O_2), more than 500 genes are up-regulated, and their induction is dependent of Sty1 and, some of them, of Atf1

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(Chen, et al. 2003, Chen, et al. 2008). However, in spite of the attempts to characterize the downstream targets of Sty1-Atf1 pathway, such as the SAGA complex (Sanso, et al. 2011), the role of the Sty1 kinase activity on the RNA polymerase II dependent transcription of stress genes is unclear. In a first hypothesis, as it has been described for the ortholog kinase HOG1 of *Saccharomyces cerevisiae* (Brewster and Gustin 2014, Hohmann 2015, Krantz, et al. 2006), Sty1 regulates the transcriptional machinery using Atf1 as an anchor to the stress promoters (Gaits, et al. 1998, Lawrence, et al. 2007, Reiter, et al. 2008); Atf1 phosphorylation could be dispensable. A second possibility is that, once at the nucleus, activated Sty1 phosphorylates Atf1 enhancing its affinity for stress promoters, since by chromatin immuno-precipitation (ChIP) there is a modest recruitment of Atf1 to stress promoters after stress imposition (Reiter, et al. 2008, Sanso, et al. 2008); and it was also revealed through ChIP-sequencing of total immuno-precipitated Atf1 that binding of Atf1 to DNA is enhanced upon activation by Sty1 (Eshaghi, et al. 2010). The last proposal is that Sty1 could be inhibiting Atf1 ubiquitin-dependent degradation (Lawrence, et al. 2009, Lawrence, et al. 2007), so that stable, phosphorylated Atf1 would accumulate and its recruitment to promoters would enhance. Another characteristic to consider in this regulatory cascade is that the expression of *atf1* gene is up-regulated 4-fold upon oxidative stress (Chen, et al. 2003, Chen, et al. 2008), which could explain enhanced protein levels after stress imposition and further recruitment of the transcription factor at promoters. Thus, in order to characterize the role of Atf1 phosphorylation by Sty1, we recently expressed HA-tagged wild-type Atf1 and different mutants at the eleven serine/threonine-proline (S/TP) phospho-sites from a constitutive promoter (Salat-Canela, et al. 2017). Cells expressing non-phosphorylatable Atf1 mutants displayed defects in the transcription of a subset of genes that are also dependent on Pap1 transcription factor, while expression of phospho-mimicking Atf1 mutants triggered constitutive transcription of a second subset of genes (Pap1-independent), even in cells lacking Sty1 MAP kinase (Fig. 1) (Salat-Canela, et al. 2017).

The characterization of Atf1 phosphorylation mutants reveals two distinct subsets of stress genes

Our study has experimentally demonstrated that among the eleven putative S/TP phospho sites present in Atf1, only six (Ser152, Ser172, Thr204, Thr216, Ser226 and Thr249) are important

for the activity of the transcription factor: cells expressing an mutant Atf1 protein with those six sites mutated to alanine or isoleucine are sensitive to oxidative stress, while cells expressing a phospho-mimicking Atf1 mutant, harboring six substitutions to aspartic or glutamic acid, are tolerant to stress even in the absence of Sty1. The transcriptome profiles of the Atf1 phosphorylation mutants revealed two different subsets of genes: a first group where only Atf1 phosphorylation is necessary to activate gene transcription, thus they are active in the phosho-mimicking mutant at basal conditions (*hsp9* and *gpd1*-like genes) and a second group of genes that still requires stress conditions to be transcribed yet they also rely on active Pap1, another bZIP transcription factor (*ctt1* and *srx1*-like genes). By ChIP experiments we have shown that Atf1 is already bound to the first subset of genes and phosphorylation is only important to promote transcription initiation (see below), while in the second group of genes a moderate binding of Atf1 to gene promoters is detected at basal conditions but the activation of Pap1 by H_2O_2 and the subsequent entry of this transcription factor at promoters facilitates further recruitment of Atf1 (Fig. 1). The hypo-phosphorylation mutants cannot activate Pap1-, Atf1-dependent genes such as *srx1* or *ctt1.* However, we cannot explain why these mutants are still able to trigger transcription of the Atf1-dependent subset of genes, exemplified by *gpd1* and *hsp9,* in a Sty1-dependent manner, and we envision several possibilities. First, we have expressed the partially active HA-Atf1.10M transcription factor in cells lacking other bZIP transcription factors (Pap1, Atf21 or Pcr1) to test whether the role of unphosphorylatable Atf1 is to drag Sty1 to promoters, which would then trigger phosphorylation of adjacent transcription factors; nevertheless, none of the combinations abolished gene transcription. Second, in order to test whether Sty1 recruitment to these promoters by HA-Atf1.10M can also promote transcription activation *per se*, we have artificially dragged Sty1 to a modified gene promoter (carrying an Atf1-to-GAL4 binding site substitution) by fusing the kinase to the GAL4 DNA binding domain (GAL4bd); while the chimera was able to activate transcription, the presence of mutant Atf1 in cells was required, suggesting that Atf1, dragged to promoters by GAL4bd-Sty1, was providing the transcription activation role. A third possibility is that Sty1 phosphorylates Atf1 at non-canonical sites; by phosphoproteomic analysis of Atf1 purified from stressed or unstressed cells, we discovered several serine and threonine residues that become phosphorylated in Atf1 after stress imposition; however, expression of a mutant lacking all those

sites (HA-Atf1.17M mutant) is still capable of promoting expression of *gpd1* and *hsp9*. Therefore, further work will be required to show how transcription is activated at these genes when Atf1 lacks all the putative phosphorylation sites.

Five out of six essential phosphorylation sites lie on the same Atf1 domain

We modeled the structure of Atf1 with the suite I-TASSER (Yang, et al. 2015), and defined several domains in this transcription factor: a trans-activation domain, and spacer domain and the DNA binding region. Out of the six S/TP phospho sites which we identified as being essential for Atf1 role as a transcription factor *in vivo*, only five are located in the same solvent-exposed protein surface constituting what we called the trans-activation domain, physically separated from the bZIP DNA binding domain by an intermediate region (Salat-Canela, et al. 2017). Interestingly, the group of Wahls analyzed the role of Atf1 on meiotic recombination at hotspots, another function of the Sty1-Atf1 cascade (see below), and based on the analysis of truncated mutant derivatives the authors defined a recombination activation domain encompassing residues 151 to 225, and therefore excluding Thr249 from an activation role (Gao, et al. 2008). Construction of an Atf1.5M mutant, lacking the Thr249-to-Ile substitution, will be helpful to confirm that this residue is not important for Atf1 activity. The presence of an intermediate (or spacer) domain between the phospho sites and the bZIP domain in our modeled structure was key to reinforce our experimental data, since the gain of negative charges upon phosphorylation at the transactivation domain is unlikely to have an effect on the distantly located DNA binding domain; in fact, this intermediate domain contains an unusual high number of positively charged residues which would promote the binding to DNA while counteracting the phosphorylation-mediated negative charges.

Specific role of Atf1 phosphorylation by Sty1 on transcription initiation

Concerning the role of Atf1 phosphorylation by Sty1, it has been previously suggested that it protects Atf1 from degradation, so that enhanced levels of Atf1 promote further recruitment of the transcription factor to gene promoters (Lawrence, et al. 2009, Lawrence, et al. 2007). Some studies also speculated that Sty1 may participate directly on transcription initiation/elongation, thus its recruitment to gene promoters by Atf1 would be vital for gene expression (Gaits, et al.

1998, Lawrence, et al. 2007, Reiter, et al. 2008). Our experimental design challenges the preceding models, and we propose that the differences arise from the following: first, we have expressed Atf1 from a constitutive promoter, abolishing the feed-back regulation of Atf1 synthesis by active Atf1; and second, we have tagged Atf1 with HA and used antibodies against the tag instead of polyclonal antibodies against Atf1, shown to recognize phosphorylated and unphosphorylated Atf1 with different affinities (Salat-Canela, et al. 2017). First, according to our model Atf1 protein levels remain constant after stress imposition, at least at short times; second, Atf1 phosphorylation *per se* is sufficient for transcription of the Atf1-dependent subset of genes, as demonstrated with the use of Atf1 phospho-mimicking mutants; and third, Atf1 is bound to Atf1-dependent gene promoters before stress imposition and phosphorylation does not increase promoter occupancy but rather promotes the recruitment of the transcriptional machinery (Salat-Canela, et al. 2017).

We are currently investigating the specific participation of phosphorylated Atf1 in the inhibition of repressor complexes, in the recruitment of activators, or in both. In fact, Degols and Russell already proposed the dual capacity of Atf1 to repress the *ctt1* gene at basal conditions and to activate its transcription upon stress, based on the enhanced levels of *ctt1* mRNA in *Δatf1* cells (Degols and Russell 1997). Genome-wide studies using microarrays have furthered reinforced this notion, since more than 4% of the *S. pombe* genes are de-repressed by more than 1.5-fold in *Δatf1* cells (Chen, et al. 2008, Sanso, et al. 2008).

Regarding the interaction of unphosphorylated Atf1 with transcriptional repressors under basal conditions, the co-repressors Tup11 and Tup12 have been reported to restrain the expression of the gluconeogenesis gene *fbp1* (Asada, et al. 2015, Takemata, et al. 2016). Atf1 has also been described to recruit repressive activities at the mating locus through the binding to several hetereochromatin assembly factors such as Clr3, Clr4, Clr6 and Swi6 (Jia, et al. 2004, Kim, et al. 2004, Yamada, et al. 2005). Regarding the putative recruitment of transcriptional activators by phosphorylated Atf1 after stress imposition, the Gcn5 histone acetyl transferase-containing SAGA complex has been reported to participate in nucleosome eviction at stress genes, and activation of Atf1 by Sty1 is required for its recruitment (Sanso, et al. 2011); activated Atf1 may directly interact with one subunit of the complex. As will be reviewed below, Sty1 and Atf1 are also required to mediate recombination at the *ade6-M26* hotspot. For

this process to occur, it has been reported that Atf1 should recruit, directly or indirectly, the SAGA subunits Gcn5 and Ada2, and other chromatin modulators such as Snf22, Hrp1, Hrp3 and Mts2, to remodel chromatin and allow the access of the recombination machinery (Hirota, et al. 2008, Yamada, et al. 2004). Future work will help us elucidate whether unphosphorylated Atf1 binds to some of these repressive complexes under basal conditions and to some co-activators after phosphorylation at the stress gene promoters.

Participation of Sty1 and Atf1 in other biological processes

Apart from their essential role in the activation of gene transcription under stress conditions, Sty1 and Atf1 have also been implicated in other cellular processes (Fig. 2). In fact, Sty1 but not Atf1 has been previously linked to cell cycle regulation under normal and stress conditions (Millar, et al. 1995, Shiozaki and Russell 1995). Sty1 has been proposed to phosphorylate diverse substrates involved in the control of mitosis and cell size such as Srk1 (Lopez-Aviles, et al. 2005, Lopez-Aviles, et al. 2008) and Plo1 (Petersen and Hagan 2005, Petersen and Nurse 2007); according to these studies the Atf1 phosphorylation mutants should not participate in cell cycle regulation.

Sty1 and Atf1 also participate in several other biological processes (Fig. 2), such as recombination at some hot spots. The *M26* allele of the *ade6* gene has a single base pair substitution which generates a CRE-like DNA site where the dimer Atf1-Pcr1 binds and promotes meiotic recombination (Wahls and Smith 1994), although this binding seems to be independent of Atf1 phosphorylation (Gao, et al. 2008). The Sty1-Atf1 pathway also has an important role in the initiation of the mating and meiosis programme by regulating the *ste11* gene upon nitrogen depletion (Kato, et al. 1996), as well as in the induction of *fbp1* transcription after glucose deprivation (Hoffman and Winston 1991). In both cases, the cAMP-dependent Pka1 kinase has to be inactivated by Cgs1 (Shiozaki and Russell 1996). However there are not CRE sites at the *ste11* promoter, and neither Atf1 nor Sty1 have been identified by ChIP at the promoter of this gene (Eshaghi, et al. 2010); Sty1-Atf1 could be regulating this process indirectly, as proposed by the group of Wahls (Davidson, et al. 2004). Life span promotion under glucose starvation is another process where Sty1 and Atf1 are involved. Glucose-limiting conditions favour reactive oxygen species production and Sty1-Atf1 pathway activation, thus

promoting lifespan extension (Zuin, et al. 2010a, Zuin, et al. 2010b). Finally, Atf1 regulates the establishment of heterochromatin at the *mat* locus in addition to the RNAi-dependent pathway by binding of Atf1-Pcr1 heterodimer to two CRE sites next to the *mat3* locus (Jia, et al. 2004). Atf1 binding to those CRE sites favours the recruitment of factors involved in heterochromatin formation such as the histone deacetylase Clr3, the methyltransferase Clr4 and the HP1 chromodomain protein Swi6. Future work will be required to test whether the phospho-mimicking Atf1 mutants require the presence or not of Sty1 in these biological processes.

Conclusions and future perspectives

As reviewed here, the main role of Sty1 in the stress transcriptional response is to phosphorylate Atf1 allowing the recruitment of the transcriptional machinery; therefore Sty1 implication in Atf1 stabilization and on transcription initiation or elongation have been ruled out. Nevertheless, many questions remain unanswered. For instance, the phosphorylation of Atf1 may create a structural platform that could attract or repel some unknown co-activators or co-repressors, respectively, and those interactions have to be biochemically characterized. Furthermore, our experiments with the hypophosphorylation mutants point towards the existence of an alternative mechanism to trigger gene transcription in the case of an Atf1-dependent subset of genes (*gpd1* and *hsp9*-like genes). Finally, Sty1 and Atf1 participate in other processes always linked to the binding of Atf1 to specific sites in the genome. Forthcoming experiments using the phosphorylation mutants will allow us to infer the importance of Atf1 phosphorylation in each of these processes.

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FIGURE LEGENDS

Fig. 1. Model for the role of Sty1 and Atf1 in the activation of stress genes. **a** Role of Atf1 at the *gpd1* and *hsp9* promoters. Non-phosphorylated Atf1 is bound to the CRE sites at the *gpd1* and *hsp9* promoters prior to stress. Phosphorylation of the transcription factor (TF) by Sty1 facilitates the recruitment of the transcriptional machinery (Pol II) and the activation of these stress genes. **b** Role of Atf1 at the *ctt1* and *srx1* promoters. Non-phosphorylated Atf1 is bound to the Pap1-dependent *ctt1* and *srx1* promoters, although to a lesser extent (lower promoter occupancy) than to the *gpd1* and *hsp9* genes. Upon H_2O_2 stress, the transcription factor Pap1 is oxidized and then binds to the AP-1 sites of these genes in an Atf1-independent manner. Pap1 binding at promoters is required for further Atf1 recruitment to the CRE sites. Synergy between oxidized Pap1 and phosphorylated Atf1 is required for efficient Pol II recruitment and full and sustained transcription activation of this subset of genes.

Fig. 2. Biological functions of the MAP kinase Sty1 and the transcription factor Atf1. **a** Scheme depicting the Sty1 cascade, which in response to environmental stresses leads to transcription up-regulation of two subsets of genes (Pap1-Atf1-dependent or only Atf1-dependent). **b** Scheme showing other biological processes regulated by Sty-Atf1. Some of them are linked to gene transcription such as survival at stationary phase (Chron. Aging), adaptation to glucose depletion (*fbp1* gene) or to nitrogen starvation (*ste11* gene); the last two are also dependent on the Pka1 pathway. Establishment of heterochromatin at the *mat* locus and recombination at the *ade6-M26* hotspot are not directly linked to transcription activation.

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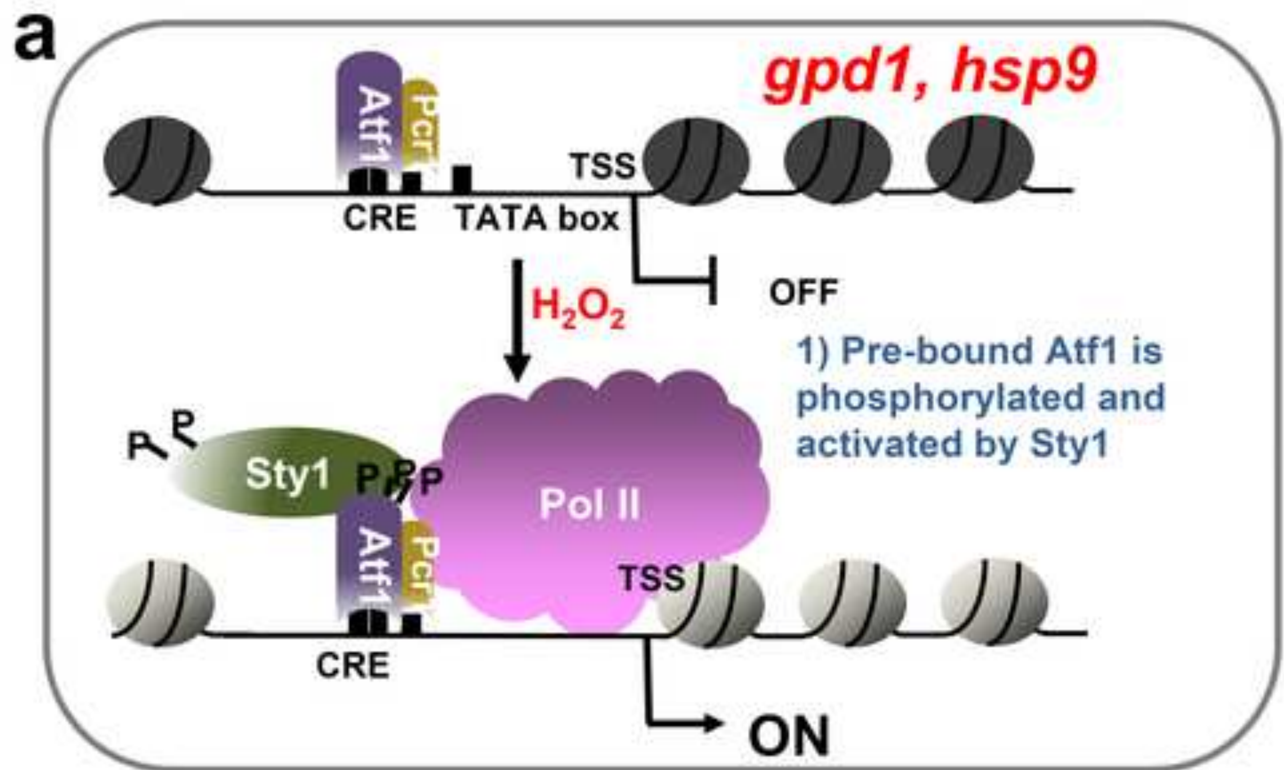

Figure 2

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